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Isolation and Characterization of Several Siderophore Producing Bacteria from Cotton Plant

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ABSTRACT: Siderophores are defined as relatively low molecular weight, ferric ion specific chelating agents synthesized by bacteria, actinomycetes and fungi growing under low ionic stress. In recent era, siderophores have received much attention because of their potential roles and applications in various areas of environmental research. In our study, *Gossypium hirsutum* samples were collected from four different sampling sites rhizospheric soil (rhizosphere, rhizoplane, endorhizosphere) and nonrhizospheric (bulk soil for comparison) from Agriculture farm, cotton research centre, Surat, Gujarat. Siderophore production was mainly *Azotobacter species*, *Pseudomonas species* and *Enterobacter species*. Microbial siderophores acts as a potential biocontrol agent against harmful phyto-pathogens and holds the ability to substitute hazardous pesticides.

KEYWORDS : Rhizosphere , siderophore, biocontrol

I. INTRODUCTION

Iron (Fe) is an essential micronutrient for the growth of almost all living microorganisms because it acts as a cofactor in enzymatic processes, oxygen metabolism, electron transfer, and DNA and RNA synthesis^[1]. Fe is also essential for biofilm formation because it regulates surface motility and stabilizes the polysaccharide matrix^{[23][9]}. Under iron-deficient conditions, the microbial surface hydrophobicity decreases. So that, it alters the bacterial surface protein composition which leads to limitation of biofilm formation^[21]. Thus, because of the low bioavailability of Fe in the environment, microorganisms have developed specific uptake strategies such as production of siderophores.

Siderophores are metal-chelating agents with low molecular masses (200–2000 Da) that are produced by microorganisms and plants^[19], Marine organisms such as phytoplankton^[22], cyanobacteria^[4] especially under Fe-limiting conditions can produce siderophores.

The role of siderophores is primarily to scavenge Fe, but they also form complexes with other essential elements ((i.e. Mo, Mn, Co and Ni) in the environment and make them available for microbial cells^{[5][8]}. Siderophores has been successfully used for bioremediation of contaminated soils by solubilizing metals^[2].

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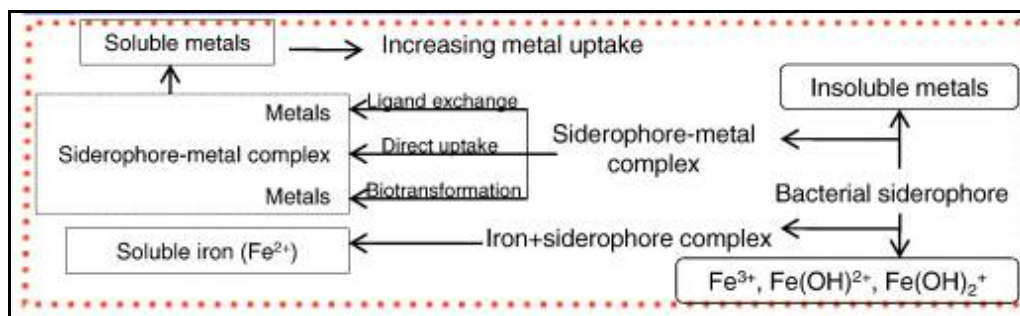


Figure – 1 : Siderophore production by rhizobacteria ^[20]

Siderophores are divided into three main families depending on the characteristic functional group, i.e. hydroxamates, catecholates and carboxylates. More than 500 different types of siderophores are known, of which 270 have been structurally characterized.

Microbial siderophore:

Microorganisms produce a wide range of siderophores. Most of the bacterial siderophores are catecholates like enterobactin, and some are carboxylates like rhizobactin and hydroxamates like ferrioxamine B. However, there are also certain types of bacterial siderophores that contain a mixture of the main functional groups like pyoverdine^[10].

Biotechnological application of microbial siderophore:

Microbial siderophores provide Fe to plants to enhance their growth when the bioavailability of Fe is low in the soil^[11]. It has significant role in the biological control mechanism as competitors for Fe in order to reduce the Fe availability for the phytopathogens^{[6][14]}. Siderophores are also extremely effective in solubilizing and increasing the mobility of dimetals Cd^{2+} , Cu^{2+} , Ni^{2+} , Pb^{2+} and Zn^{2+} ^[3]; trivalent cations, such as Mn^{3+} , Co^{3+} and Al^{3+} ; and actinides, such as Th^{4+} , U^{4+} and Pu^{4+} ^[16] in metal bioremediation^[17]. Siderophores have been shown to be an environmentally friendly alternative to hazardous pesticides^[18]. Thus siderophores have potential ability to resolve various environmental problems like heavy metal accumulation, rust removal, biofouling, dye degradation, sewage treatment and bioleaching.

II. MATERIALS & METHOD

Sample collection:

The soil samples were collected from *Gossypium hisrsutum* (wild cotton, transgenic cotton variety 1 (Bt1) and transgenic cotton variety 2 (Bt2)) at different site of Agriculture farm, Cotton research centre, Surat. In monsoon and winter seasons were selected for the isolation of plant growth promoting rhizobacteria (PGPR).

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Figure– 2:A.Wild Cotton, B. transgenic cotton variety 1(Bt1), C.transgenic cotton variety 2(Bt2)

Isolation of PGPR:

The selective media such as Pikovskaya agar medium, Nitrogen free medium, Bacillus medium, Ashby's mannitol agar, King's B medium, Yeast extract mannitol agar were used for isolation of PGPR. All the inoculated plates were incubated for appropriate duration and visible growth were reported as their growth characteristics. All the isolates were coded considering selective media / sample plant / sample site / number. The Gram staining and motility test were performed as primary screening. These bacterial cultures are maintained on respective media on slant as per the standard procedure at 4 °C as well as in 10 % glycerol broth for longer duration and further study.

In Vitro screening of Siderophore production of rhizobacteria (PGPR):

Siderophore study is carried out mainly for two zones such as nonrhizospheric (Bulk soil) and rhizospheric zone (rhizosphere, rhizoplane and endorhizosphere). The isolate were screened for siderophore production by qualitative as well as quantitative assay.

Qualitative detection of siderophore (Plate assay):

Siderophore production was tested qualitatively using Chrome azurol S (CAS) agar as described by Schwyn and Neilands. CAS agar was prepared from four solution which were sterilized separately before mixing. CAS plates were streaked with bacterial strains and observed for development of orange halo against dark blue background around the colonies after 48-72 h of incubation at 28°C. A change in color from blue to orange (hydroxamate type siderophore) or purple (catechol type siderophore) was considered as a positive reaction^[19].

Quantitative spectrophotometric assay for siderophore production (Liquid assay):

Cultures were grown in a selective media at 37 °C for 24 hours under shaker conditions (100 rpm). The cells were removed by centrifugation at 3000 rpm for 15 minutes. 0.5 ml of the culture supernatant was then mixed with 0.5 ml CAS solution and 10µl shuttling solution (sulfosalicylic acid). The color obtained was determined using the spectrophotometer at 630 nm after 20 minutes of incubation. Reference solution (Medium without inoculation+ CAS dye + shuttle solution) were used as standard during the determination.

Characterization of PGPR:

Colonial and morphological diversity of bacteria were used for identifying PGPR. Among them, Gram reaction of bacteria were performed as differentiating test. Motility was determined by TTC test using TTC semi solid broth^[13]. Stabing was done on this semisolid broth media with all bacteria isolates and incubate at room temperature for 3 days. The PGPR having potential for multifunctionalities were phenotypically identified using various biochemical test namely biochemical reaction (Indole Production test, Methyl Red test, Voges-Proskauer's test, Citrate production,

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Nitrate reduction test, H₂S Production), sugar and sugar alcohol fermentation (Glucose, Lactose, Sucrose, maltose, mannitol, Xylose, Fructose, Arabinose, Inositol), enzyme profile (Phenylalanine Deaminase, Gelatinase, Catalase, Urease, Amylase, Lipase) and confirmative test (Triple sugar Iron, Lysine Iron decarboxylase, Oxidation/Fermentation test) were tested for the multitrait PGPR^[7].

III. RESULT AND DISCUSSION

The main focus of this study is on the screening of a potential siderophore producing PGPR strains indirectly promoting production of inhibitory substance which prevents the deleterious effect of plant pathogen on plant or increase the natural resistance of host.

Rhizospheric microflora on selective media:

Total 332 bacterial species were isolated from the four different sites – bulk soil, rhizosphere, rhizoplane, endorhizosphere of wild and transgenic cotton (transgenic cotton variety -1 [Bt-1] and transgenic cotton variety -2 [Bt-2]) from cotton research centre, Surat. Majority rhizobacteria were Gram negative, motile short rods.

Considering the number of PGPR, the diverse group of rhizobacteria were screened from the rhizosphere of cotton plant. Maximum number of *Pseudomonas sp.* were present in this field and are important for plant growth promoting traits. Similar study was done by Rezzonico and his co-worker in 2007 concluded that *Pseudomonas*, is one of the most common bacterial species of the rhizosphere. Other PGPR species present in rhizosphere and nonrhizosphere region were *Bacillus species* > *Aeromonas species* > *Azotobacter species* > *Enterobacter species* > *Alcaligen species*. (Figure 3)

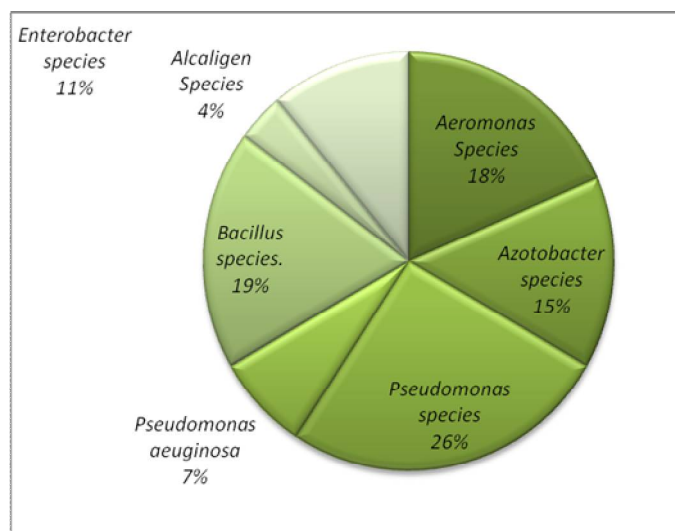


Figure – 3: Diverse bacterial species in field

Siderophore production:

Bacteria produce siderophores as iron chelating agents especially during iron deficiency including *Bradyrhizobium*, *Pseudomonas*, *Rhizobium*, *Streptomyces*, *Serratia* and *Azospirillum*^[23]. Inoculation with siderophore-producing bacteria grown under iron limiting conditions has a positive effect on plant growth. It was found that 27 isolates such as *Azotobacter species*, *Pseudomonas species*, *Enterobacter species*, *Aeromonas species*, *Bacillus species*, *Alcaligen species* were recorded after 3-4 days of incubation which showed positive for siderophore production (Figure 4). All of these species *Enterobacter sp* isolated from rhizoplane of transgenic variety 1 cotton plant produced large amount of siderophore production.

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Sr. No.	Siderophore producing PGPR		Estimation of Siderophore at 630 nm
1	NFM/W/BS/2	<i>Aeromonas Species</i>	0.512
2	AMA/W/BS/2	<i>Azotobacter Species</i>	0.633
3	KM/W/BS/2	<i>Pseudomonas species</i>	0.251
4	KM/W/BS/3	<i>Pseudomonas species</i>	0.267
5	KM/W/BS/4	<i>Pseudomonas species</i>	0.291
6	BM/W/BS/1	<i>Bacillus Species</i>	0.546
7	BM/W/BS/3	<i>Bacillus Species</i>	0.342
8	NFM/W/RH/2	<i>Aeromonas Species</i>	0.234
9	AMA/W/RH/2	<i>Azotobacter Species</i>	0.613
10	KM/W/RH/3	<i>Pseudomonas aeuginosa</i>	0.243
11	KM/W/RH/4	<i>Pseudomonas Species</i>	0.199
12	BM/W/RH/2	<i>Bacillus species</i>	0.721
13	BM/W/RH/3	<i>Bacillus species</i>	0.918
14	AMA/W/RP/2	<i>Azotobacter species</i>	0.293
15	AMA/W/RP/3	<i>Azotobacter species</i>	0.724
16	KM/W/RP/3	<i>Pseudomonas species</i>	0.534
17	KM/W/RP/4	<i>Pseudomonas species</i>	0.265
18	BM/W/RP/3	<i>Bacillus species</i>	0.652
19	KM/W/ER/4	<i>Pseudomonas species</i>	0.618
20	YMA/BT1/BS/7	<i>Aeromonas species</i>	0.523
21	NFM/BT1/RH/2	<i>Aeromonas Species</i>	0.430
22	NFM/BT1/RP/2	<i>Alcaligen Species</i>	0.212
23	YMA/BT1/RP/7	<i>Aeromonas species</i>	0.960
24	YMA/BT1/RP/11	<i>Enterobacter species</i>	1.593
25	YMA/BT1/ER/11	<i>Enterobacter species</i>	1.042
26	BM/BT2/RH/2	<i>Enterobacter species</i>	1.165
27	KM/BT2/RP/3	<i>Pseudomonas aeuginosa</i>	0.256

Table 1: PGPR and siderophore production

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PGPR were identified by various biochemical tests as per the following Table 2:

Isolate code		NFM/W/BS/2 NFM/W/RH/2 YMA/BI/BS/7 NFM/BI/RH/2 YMA/BI/RP/7	AMA/W/BS/2 AMA/W/RH/2 AMA/W/RP/2 AMA/W/RP/3	KM/W/BS/2 KM/W/BS/3 KM/W/BS/4 KM/W/RH/4 KM/W/RP/3 KM/W/RP/4 KM/W/ER/4	KM/W/RH/3 KM/BI/2/RP/3	BM/W/BS/1 BM/W/BS/3 BM/W/RH/2 BM/W/RH/3 BM/W/RP/3	NFM/BI/RP/2	YMA/BI/RP/11 YMA/BI/ER/11 BM/BI/2/RH/2
Biochemical reaction	IN	+	-	-	-	-	-	-
	MR	+	-	-	-	+	+	-
	VP	-	+	+	+	-	-	+
	CT	+	+	-	-	-	+	+
	NR	+	+	+	+	+	+	+
	H ₂ S	+	+	-	-	-	-	-
Sugar and sugar alcohol fermentation	Glu	+	+	-	+	+	+	+
	Lac	-	-	-	-	+	-	+
	Suc	+	+	-	NG	+	-	+
	Mal	+	+	NG	NG	NG	NG	+
	Man	+	+	NG	NG	-	-	+
	Xyl	NG	NR	NG	NG	NG	NG	+
	Fru	NG	NG	NG	NG	NG	+	NG
	Ara	NG	NG	NG	NG	-	NG	NG
Ins	NG	NG	-	-	NG	NG	NG	
Enzyme profile	Pa	-	-	-	-	-	-	-
	Gel	-	-	+	+	+	+	-
	Cat	+	+	+	+	+	+	+
	Ura	-	N	-	-	-	-	-
	Amy	+	+	-	-	+	+	-
	Lip	-	-	-	+	-	-	-
TSI	Slant	+	+	+	+	-	+	+
	Butt	-	+	-	-	+	+	+
	H ₂ S	-	+	-	-	-	+	-
	Gas	-	-	+	+	-	-	+
LYD	Butt	-	+	+	+	+	-	+

Key :

***Biochemical reaction** : IN– Indole Production test , MR – Methyl Red test , VP – Voges-Proskauer’s test , CT – Citrate production , NR – Nitrate reduction test , H₂S – H₂S Production ,

***Sugar and sugar alcohol fermentation** : Glu- Glucose , Lac- Lactose , Suc- Sucrose , Mal – Maltose , Man- Mannitol , Xyl – Xylose , Fru- Fructose , Ara – Arabinose , Ins – Inositol

* **Enzyme profile** : Pa – Phenylalanine deaminase , Gel – Gelatinase Cat – Catalase , Ura – Urease , Amy – Amylase , Lip – Lipase

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***Conformative test** : TSI – Triple sugar Iron, LYD- Lysine decarboxylase, O/F – oxidative fermentation)
*NG- Not growth, NR- Not require , ND – Not determine, BS- Bulk soil, RH- Rhizosphere , RP- Rhizoplane, ER – Endorhizosphere

Table - 2: Characterization of PGPR

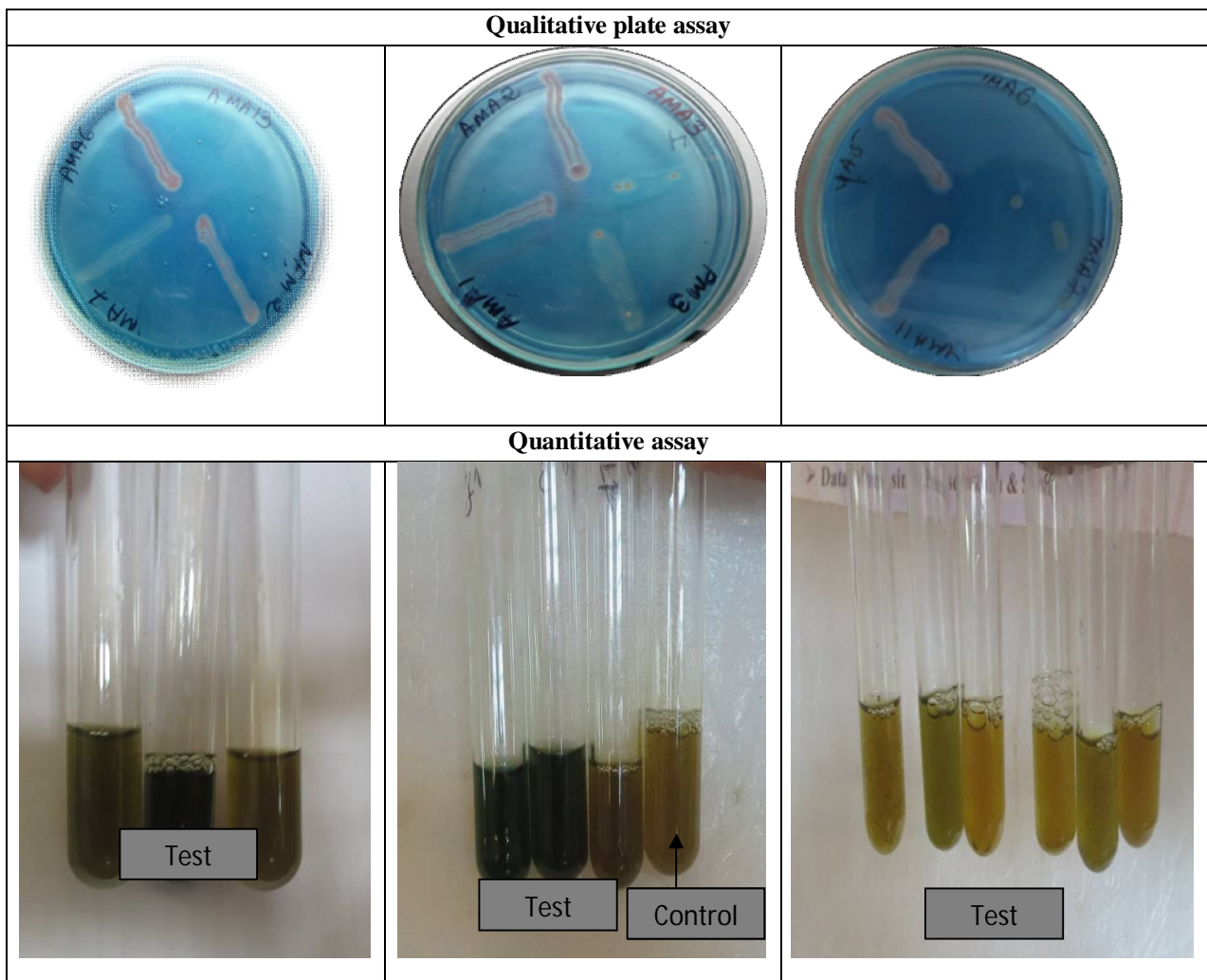


Figure 4 : Siderophore production by PGPR

IV. CONCLUSION AND FUTURE SCOPE

The study of mineral–microbe interactions underscores the importance of microorganisms in making the earth a suitable environment for all forms of life. Iron is a vital element required by every living organism for numerous cellular processes. Under iron-deficient conditions, the growth of microorganisms becomes impaired. Microorganisms

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survive under such iron-limited conditions by secreting siderophores. It has become clear that siderophores represent central organic compounds in Fe uptake in many microorganisms. From the present study it can be concluded that rhizosphere soil organism producing siderophore is used to improve the fertility of the soil to help the plant grow well and increase the Fe content of the plants and good antibacterial activity for phytopathogenic organisms. It was found that different siderophore producing bacteria produced siderophore at different time and with different percentage. The importance of siderophores is playing a significant role in the environmental applications. In medicine, Siderophores have applications for iron and aluminum overload therapy such as β -thalassemia, Anti-Malaria drug and antibiotics for improved targeting. More research has been focussed on finding effective ways to use siderophores in ecology, agriculture, bioremediation, biosensor and biocontrol, which should enhance their application in the environment

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